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In Vivo Screening of S100B Inhibitors for Melanoma Therapy

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Abstract

S100 proteins are markers for numerous cancers, and in many cases high S100 protein levels are a prognostic indicator for poor survival. One such case is S100B, which is overproduced in a very large percentage of malignant melanoma cases. Elevated S100B protein was more recently validated to have causative effects towards cancer progression via down-regulating the tumor suppressor protein, p53. Towards eliminating this problem in melanoma, targeting S100B with small molecule inhibitors was initiated. This work relies on numerous chemical biology technologies including structural biology, computer-aided drug design, compound screening, and medicinal chemistry approaches. Another important component of drug development is the ability to test compounds and various molecular scaffolds for their efficacy in vivo. This chapter briefly describes the development of S100B inhibitors, termed SBiXs, for melanoma therapy with a focus on the inclusion of in vivo screening at an early stage in the drug discovery process.

Keywords

In vivo screening; Preclinical testing; Intratumoral delivery; Systemic delivery; Pharmacokinetics; Pharmacodynamics; Maximum tolerated dose; Therapeutic window; Genetically modified mouse models; S100 proteins; EF-hand

1. Introduction

In malignant melanoma (MM), the tumor marker S100B binds directly to wild-type p53, dissociates the p53 tetramer, enhances hdm2-dependent ubiquitination of p53, and down-regulates p53-dependent tumor suppression functions (1–3). As a proof of principle for drug design, inhibiting S100B with small interfering antisense RNA (siRNAS100B) or with several S100*B inhibitors* (SBiXs; X = compound number) was achieved and shown to restore wild-type p53 at the protein level. Inhibiting S100B production was also found to cause an increase in the levels of p53 gene products as necessary to induce cell growth arrest and apoptosis in malignant melanoma (2, 4–7). Also encouraging from a drug development standpoint is that ablation of S100B expression in mice via gene targeting produces unremarkable phenotypes with few, if any, problematic physiological consequences (8–13).

With any drug-design program, there is a need to obtain physiological data at an early stage in the process to help determine whether a compound or a series of compounds induce off-target effects and/or cause other unanticipated toxicities. This is particularly important for S100 inhibitors since there are over 20 structurally similar proteins in the S100 protein family, and they each regulate several physiologically important pathways in a cell-specific manner (14, 15). Thus, an S100 inhibitor could have multiple phenotypes depending on the

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[5]Avertin has been reported to be an irritant and the following steps should be taken to minimize decomposition: (1) store and use under sterile conditions; (2) store in the dark at 4°C; (3) adjust pH of solution to >5.0; (4) discard stock solution after 4 months; (5) discard working solution after 7 days; and (6) discard any solutions that become discolored or have precipitate.

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number of S100 proteins it blocked and the S100 status of the cell-type targeted. In the case of blocking the S100B–p53 protein–protein interaction in malignant melanoma, there exists a separate issue with regard to a feedback loop that is initiated when S100B is inhibited since the gene for S100B itself is up regulated by the tumor suppressor protein, when p53 levels are restored (2). To address these and other issues, this chapter describes the discovery/development of SBiXs for melanoma therapy with a focus on the importance of doing in vivo screening of lead compounds at an early stage in the drug development process. It is likely that such an approach of performing early in vivo screening could benefit many drug discovery programs.

Small molecule inhibitors have been reported for numerous S100 family members, some of which are in clinical trials. Inhibitors of the S100A10–annexin A2 interaction have not been validated in vivo but their predicted clinical uses include angiogenesis and cancer metastasis therapy (16, 17). The anti-allergic drug cromolyn disrupts S100P–RAGE interaction and reduces pancreatic tumor formation in animal models (18). Interestingly, cromolyn also binds other S100 family members (S100A1, S100A12, and S100A13), but its effects on the interaction of these family members with their target proteins have not been fully investigated. Other small molecules that bind S100A1 include pentamidine and propanolol (19). In the case of S100A4, several phenothiazines block S100A4-mediated depolymerization of myosin-IIA filaments (20, 21). S100A4 also binds anti-allergic drugs and a modified version of azaxanthone, which is in clinical trials for treatment of metastatic disease states (21, 22). Two SBiXs developed by our group disrupt S100B–p53 complex formation and prevent unregulated melanoma cell growth and are currently being tested in human and veterinary clinical trials as potential melanoma therapeutics.

For the development of SBiXs, a combination of computer-aided drug design (CADD), high-throughput screening (HTS), structural biology, medicinal chemistry, and in vivo biology/drug testing approaches is employed. The benefits of a structure-based approach are taken advantage of for developing new compounds as well as addressing issues with regard to target specificity since the 3D structures for several S100 and S100–target complexes are published (4, 15, 19, 23–27). Likewise, structure/activity relationships (SAR) and SAR by NMR approaches are more efficiently directed when the structures are available (28, 29). This is especially important for the difficult hurdle of inhibiting a protein–protein interaction such as the S100B–p53 complex (4, 7, 27, 30). More specifically, modifications on a particular scaffold that depend on the structure of a lead compound bound to S100B are achieved iteratively by using CADD-directed medicinal chemistry; likewise, structural studies can be used to identify multiple sites within the p53-binding cleft on S100B via structural biology approaches (NMR, X-ray), so compounds and combinations of compounds can be rationally linked together synthetically to obtain tight and highly specific S100B binders (i.e., relative to binding S100A1 for example). Such a drug development process is very dependent on the latest new results, so this process occurs in a very iterative manner (Fig. 1). Once promising leads are identified, then it is important that they are screened for their in vivo efficacy and toxicity. Such leads are chosen based on binding affinities (K_Ds) and/or specificity both in biochemical assays (i.e., S100B thermodynamic/kinetic data versus other S100s) and in cellular assays (i.e., IC_{50}s). For the cellular assays, specificity and other potential off-target effects are evaluated by comparing IC_{50} values of isogenic cell lines plus/minus S100B present (31, 32). Of the approximately 100–200 lead compounds that undergo extensive in vitro testing each year, eight to ten typically meet the criteria for early-stage in vivo screening.

High-throughput biochemical and cellular screens identify two types of lead SBiXs: FDA-approved compounds and new chemical entities. Since there are no effective treatments for melanoma, repurposing of FDA-approved lead SBiXs for melanoma therapy is a high

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priority. Efficacy evaluation is the primary focus for repurposing approved drugs, since extensive pharmacological/toxicological information is already available for many species, including humans. In fact, phase II human and phase I canine clinical trials for two FDA-approved SBiXs are underway. However, like most drug discovery programs, the vast majority of lead SBiXs are new chemical entities that require extensive development and optimization. In vivo testing in animal models plays a fundamental role in developing new anticancer drugs. Prior to human testing, preclinical tolerability (maximum tolerated dose, MTD), pharmacokinetics (PK), pharmacodynamic (PD), and efficacy assays provide key information that is used to improve advanced leads. For example, a compound may need to be more lipophilic to pass through the cell membrane and reach its target or side groups may need to be added to allow for oral delivery or brain penetration.

In the case of accessible tumors such as melanoma, drugs can be delivered directly to the tumor (intratumoral) without optimization for systemic delivery and/or minimization of toxic effects on normal cells. In addition, intratumoral delivery can achieve significantly higher drug concentrations at the site of action than systemic delivery. In the clinical setting, intratumoral administration has been used to deliver gene therapy constructs, complex biologics, and small molecules to a variety of cancers including adenoviral based p53 genes in head and neck cancer (33); TNFalpha genes in rectal cancer (34); interleukin-2 in melanoma (35); immunostimulant CpG in brain cancers (36); single treatment of a meta-static squamous cell carcinoma lesion (37); BCNU in combination with radiotherapy in glioma (38); and para-toluenesulfonamide in non-small cell lung cancer (39). Intratumoral delivery has been used in the preclinical evaluation of siRNAs (40) and immune modulators (41, 42) as well as to reduce the toxicity of approved agents such as melphalan (43); 2-deoxy- D -glucose alone and in combination with carboplatin (44); and paclitaxel/docetaxel in mammary, bladder, prostate, and head and neck cancers (45–48). Our S100B inhibitor drug development program includes an intratumoral in vivo screen of lead SBiXs before preclinical testing of advanced leads. While in vivo testing prior to medicinal chemistry optimization and ADME testing is atypical, the availability of in vivo screening data early in the drug discovery process focuses resources on SBiXs with the greatest probability of clinical success.

A major consideration for any in vivo trial is the selection of an appropriate animal model. Mouse models are a mainstay in animal testing because it is feasible to perform studies in a short period of time. However, no single mouse model recapitulates the complex genetics and biology of human melanoma (49–52). Therefore, the biological question being asked as well as the advantages and limitations of the various types of models were important criteria in selecting an animal model for intratumoral in vivo screening of SBiXs. Syngeneic transplantation models involve the implantation of well-characterized melanoma cell lines into a syngeneic host and do not recapitulate many aspects of the human disease because characterized mouse lines do not reflect the heterogeneity observed in human tumors. Xenogeneic transplantation models involve the implantation of cell lines or patient-derived cells into an immunocompromised host. Both of these transplantation models can be flank models in which subcutaneous tumors are grown on the backs of mice or orthotopic models in which tumors are grown at the site of origin (i.e., breast cancer cells in the mammary fat pad). Orthotopic models have a better chance to metastasize but are more difficult to monitor. One disadvantage of human xenograft models is the fact that the host is immunocompromised and immunosurvelliance has been shown to play a role in limiting metastasis (53). In the case of SBiXs, S100B's effects on immune responses in the CNS are well documented although little is known about its role in peripheral immune responses. Nonetheless, human xenograft models are usually considered to be superior to genetically modified mouse models because the composition of the resulting tumor mimics the heterogeneity observed in patients. However, the development of multi-allelic genetically

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engineered mouse models that mimic spontaneous tumorigenesis and heterogeneity as well as target validation in simulated clinical trials using standard of care chemotherapeutics confirm the utility of genetically modified mouse models in developing cancer therapeutics (54).

The RAS-induced INK4a/ARF$^{-/-}$ mouse melanoma model (55) was chosen for in vivo screening of lead SBiXs because it has (1) an intact S100B–p53 signaling pathway (elevated S100B and wild-type p53), (2) an intact immune system, (3) tumors which are amenable to intratumoral delivery, and (4) a proven record in developing new melanoma therapies (55, 56). The Tyr::RAS^{G12V} / INK4a/ARF$^{-/-}$ line is bigenic and contains two genomic mutations on an FVB background: a mutated H-ras^{G12V} transgene on the Y chromosome and inactivated INK4a/ARF alleles on chromosome 4. This model is not commercially available and we maintain a breeding colony that generates experimental animals as well as breeders for the individual Tyr::RAS^{G12V} and INK4a/ ARF$^{-/-}$ lines. Founders for both lines were obtained from the National Cancer Institute Mutant Mouse Resource (Frederick, MD). At 2–3 months of age experimental Tyr::RAS^{G12V} /INK4a/ ARF$^{-/-}$ males develop spontaneous cutaneous melanomas in the pinna of the ears (30%), torso (23%), and tail (20%) without distant metastasis (55). Our intratumoral in vivo screening protocol for modified SBiXs is a longitudinal design with a study period of 3–8 weeks and uses the relative tumor proliferation rate (the tumor volume at a particular treatment interval/tumor volume at the time of treatment initiation) as the primary outcome (Fig. 2). Although this trial design is not optimized for garnering tolerability, PK or PD information, the gross/histological pathology, SBiX levels, and p53 pathway reactivation in the tumors are monitored and this information is useful in selecting advanced leads that will proceed to preclinical testing.

In the case of modified leads with predicted ADME properties favorable for systemic administration, concurrent tolerability (MTD) and pharmacokinetic (PK) assays are also conducted. These trials provide valuable pharmacological information that cannot be garnered from local administration trials including potential effects of the route of administration on tumor responsiveness (57). MTD and PK studies are conducted in the same species/strain and employ the same dosing scheme that will be used in subsequent efficacy studies. For example, if the expected SBiX dosing scheme is 2 weeks of consecutive intraperitoneal (IP) administration in female nu/nu mice, then the MTD experiment should be dosed IP daily × 14 in female nu/nu mice. The route of administration, oral (PO), intraperitoneal (IP), intravenous (IV), or subcutaneous (SC), is determined by the predicted chemical properties of the SBiX. In MTD experiments, animals are monitored post cessation of dosing for delayed toxicity for a minimum of 1 week and optimally 2 weeks. In mouse models, the best indicator for toxicity is weight. MTDs for novel cancer drugs are considered an LD_{10}, the dose at which 10% of mice in one group die or lose 20% of their body weight (58) (Fig. 3). The therapeutic index for cancer drugs is very small in that the MTD can be equivalent to the effective dose. The goal of PK studies is to select a dose and route of administration that allow the plasma/target tissue drug levels to exceed the IC_{50} value from cellular assays and/or K_D from biochemical screens. SBiX levels in the tumor tissue provide useful information about tumor penetrance, half-life in target tissue, and the compound's ability to leave the bloodstream. Also, SBiX levels in brain tissue assess brain penetration and potential for use in metastatic disease therapy and primary brain cancers. The data from the intratumoral in vivo screening, MTD, and PK trials described below determines if modified leads are suitable for preclinical testing, require additional medicinal chemistry optimization and further testing prior to preclinical testing, or are eliminated.

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2. Materials

1. SBiX stock: 100 mM in sterile dimethylsulfoxide (DMSO). Dissolve appropriate amount of SBiX in sterile- filtered DMSO to achieve final concentration of 100 mM. Prepare 100 μl aliquots and store at −20°C.
2. SBiX Injection Solution: For intratumoral in vivo screening, the SBiX injection solution composition is dependent upon the tumor volume and the K_D /IC_{50} of the SBiX, i.e., injection volume ≤ 20% of tumor volume and $[SBiX]_{intratumor}$ = fivefold over K_D or IC_{50}, whichever is greater. For systemic tolerability studies, SBiX injection solution composition is dependent upon the dose and appropriate volume range for the route of administration. Dilutions are made in sterile phosphate-buffered saline (PBS).
3. Vehicle-Only Control: Dilute sterile DMSO into PBS in the same ratios used to prepare the SBiX injection solution in item 2.
4. Avertin solution: Avertin stock solution (100%) prepared by dissolving tribromoethanol in tertiary amyl alcohol and stored in a brown glass bottle at 4°C. The avertin working solution (2.5%) is prepared fresh weekly by diluting 1.25 ml stock solution (100%) to 48.75 ml sterile filtered PBS. After the pH is adjusted to 7.4, the solution is filtered through a 0.22 micron filter and stored in a brown bottle at 4°C. All containers should be labeled in accordance with institutional and regulatory policies regarding the use of animals.
5. Syringes: 20 G × 38 mm gastric gavage needles (plastic or metal). 1 cc tuberculin syringes with 25G, 26G, 27G, or 30G needle.
6. Calipers: TTC Digital Caliper 0–150 mm.

3. Methods

All experiments involving animals described in this section were approved by the institutional IACUC and performed in accordance with the NIH guidelines for the treatment of animals. Appropriate institutional and/or regulatory agency approval is required for these procedures.

3.1. Intratumoral In Vivo Screening

Ten or more experimental Tyr::RAS^{G12V} /INK4a/$ARF^{-/-}$ males are obtained from our in-house breeding colony and randomly assigned to a cohort (experimental or control) when tumors ≥ 20 mm^3 in size develop.

1. Assess and record the general health status using the following criteria: body weight, grooming, eating, drinking, alterness/lethargy, mobility, coat (smooth vs. ruffled), and posture (hunch vs. upright) (see Note 1).
2. Assess and record the gross pathology of the tumor using the following criteria: erosion/invasion, redness, in flammation, and weeping (edema and presence of fluids) (see Note 1).
3. Restrain the animal and measure the length (l) and width (w) of the tumor (mm) with calipers (see Note 2).

[1]Any animals exhibiting clinically significant health issues should be evaluated for removal from the study.
[2]If a scruff hold blocks tumor access, then isoflurane anesthesia can be used for immobilization.

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4. Slowly inject the SBiX injection solution or vehicle-only control directly into the tumor.
5. Monitor and record the overall health status, gross tumor pathology, and tumor size daily.
6. Administer SBiX or vehicle-only control every other day. Rotate the injection site to minimize in flammation (see Note 3).
7. At the conclusion of the study period (3–8 weeks), euthanize animals using anesthetic overdose followed by decapitation. Administer 0.03 ml/g body weight of sterile Avertin (2.5% w/v) by ip injection. Administer additional doses of 0.15 ml as needed to attain a deep plane of anesthesia as determined by pedial and breathing reflexes (see Notes 4–[6]).
8. Examine and record the gross pathology of the tumor. Excise and weigh the tumor. Slice the tumor in half along the long axis. Process one-half for histological analysis (formalin fixation and paraffin-embedding) and the other half for biochemical/pharmacological analyses (snap frozen).
9. Examine internal organs for gross pathology and remove any abnormal organs for processing and histological analysis.
10. Tumor response is expressed as the mean tumor proliferation rate ± the SEM at 7, 14, and 21 days, i.e., the tumor volume ($V = ½ab^2$) on day 7, 14, or 21 divided by the tumor volume at day 0 (Fig. 2)

3.2. Systemic Tolerability Testing (MTD and PK)

MTD studies are preferably carried out in efficacy species/strain but can be performed in an outbred mouse followed by a small bridging study in efficacy species/strain. Mice should be acclimated for at least 3 days prior to start of experiment. Three mice are the absolute minimum required and more than 5 are utilized if fine-tuning of the dose is required. The dosing schedule and route of administration (PO, IP, SC, IV, or IM) should be chosen based on the desired dosing schedule/route of administration in the proposed efficacy experiment. Mice should be monitored for 1–2 weeks post cessation of dosing to monitor for long-term toxicities.

1. Divide mice into four groups of three to ten mice. Each group will receive a different dose, i.e., 10×, 5×, 2.5×, and 1× dosing. If the vehicle is known to be nontoxic it may be excluded from the MTD study.
2. Novel compounds should be dosed IP, IM, SC, or IV using dosing schedule planned for future efficacy experiment. Depending on the route of administration, mice are either restrained by hand (IP, IM, and PO) or immobilized in a mouse restrainer and the tail is warmed by infrared light, hot water, or warm ethanol (IV). Usually mice are not anesthetized for dosing (see Note 7 for dosing volumes and needle size).
3. Mice should be monitored or observed daily and weighed 3 times per week.

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[3]The trial schedule can be adjusted to match the predicted pharmacokinetics of the SBiX. Trials for SBiXs that are predicted to concentrate in tumor cells have used a 5-day on and 2-day off schedule. Trials for SBiXs that are not predicted to concentrate in tumors have used everyday and every other day schedules.

[4]The trial duration for a typical SBiX in vivo screen is 3 weeks. However, the trial duration can be shortened or lengthened. If tumors progress to complete remission and/or unexpected toxicities are encountered, then trial duration can be shortened. Trial duration for the Tyr::RAS^{G12V} /INK4a/ARF$^{-/-}$ model should not exceed 8 weeks because animals will be approaching the age (5.5 months) at which 50% of the mice succumb to tumor growth.

[6]Other approved methods of euthanasia can be used.

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4. Unacceptable toxicity is the inability of a mouse to ambulate in order to drink and eat in a 24-h period or 20% body weight loss at any time or 15% body weight loss over 72 h as compared to day 1 of the experiment. Animals that meet these criteria should be removed from the study and euthanized (see Note 8) (Fig. 3).

5. When dosing is complete, mice are monitored for 2 weeks to detect delayed toxicities.

Pharmacokinetic studies are carried out in naïve mice that may be tumor bearing or not. PK experiments are usually carried out with 18 mice (unless the desire is to test more time points).

1. Mice are divided into six groups of three mice.

2. Three mice are in the control group (time zero—no drug or vehicle) and 15 mice are in the treated groups (see Note 9) if mice will be implanted with tumor cells.

3. SBiXs are usually dosed ONCE by the route of administration planned for the efficacy experiment. The dose can range from 10 to 50 mg/kg.

4. Mice are euthanized (e.g., CO_2 inhalation) at specific time points post dosing. For SBiXs, typical time points are 15 min, 30 min, 1 h, 4 h, and 8 h post dosing (see Note 6).

5. Blood is collected by cardiac puncture (27G needle with 1 ml disposable syringe) and plasma is isolated after centrifugation in BD microtainer heparinized tubes. Mice are placed in left hand and 1 cc syringe with 27 G 1/2 ″ needle is inserted under the sternum towards the heart. The plunger is slowly pulled from the barrel. If no blood is observed, the needle is pulled out and reinserted. The needle is removed from barrel before injecting blood into microtainer tube. The tube is immediately capped and inverted several times to prevent clotting. Samples are

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Route	Needle size and volume
Oral (PO)	20G × 38 mm gastric gavage needles (plastic or metal) Dosing volume should be 10 ml/kg and no more than 5 ml/kg
Intraperitoneal (IP)	26 or 27G needle with 1 ml disposable syringe Dosing volume should be 10 ml/kg and no more than 5 ml/kg
Subcutaneous (SC)	26 or 27G needle with 1 ml disposable syringe Dosing volume should not exceed 300 ul
Intravenous (IV)	27G to 30G needle with 1 ml disposable syringe Dosing volume should not exceed 10 ml/kg
Intramuscular (IM)	27G needle with 1 ml disposable syringe 0.05 ml can be injected into calf muscle

8If one mouse in a group of three loses 20% body weight loss, this may be past the MTD since the number of animals is very small. The study would have to be repeated using the reported MTD and lower doses.

9The cell line of choice will be grown in culture using optimal conditions. Usually, 1×10^6 to 5×10^6 cells are injected with 33% to 50% Matrigel™ (BD Biosciences) on the right fl ank of mice. Cells are injected in 0.15–0.2 ml of volume with a 27G tuberculin syringe. Tumors should be monitored 3 times per week with electronic calipers as described earlier. When tumors reach approximately 400–500 mm^3, the mice can be divided into six groups of three mice and the PK study may be initiated.

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centrifuged in a tabletop centrifuge for 10 min at max speed. Plasma is poured off into labeled microfuge tube and stored at −20°C until analysis.

6. Tumor, brain, or other tissue may be collected at time points noted. Tissue is snap frozen in an ethanol/dry ice bath and stored at −80°C until analysis.

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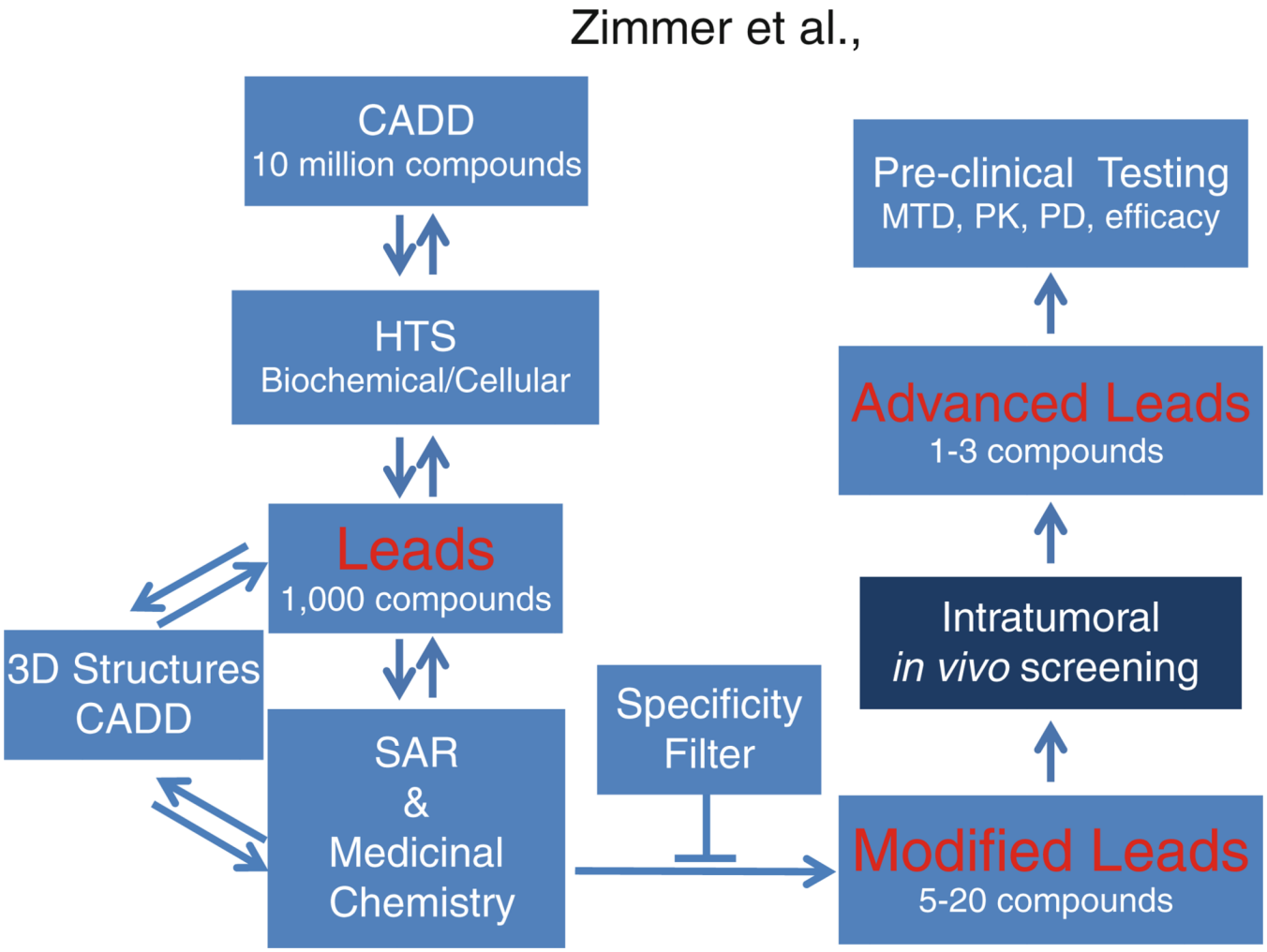

Figure 1.
Strategy for the discovery and development of SBiXs. An iterative process that includes computer-aided drug design (CADD), high-throughput screening (HTS), structure/activity (SAR) and medicinal chemistry, 3D structure (NMR and X-ray), and a specificity filter is used to engineer modified SBiXs for intratumoral in vivo screening and subsequent advanced leads suitable for preclinical evaluation.

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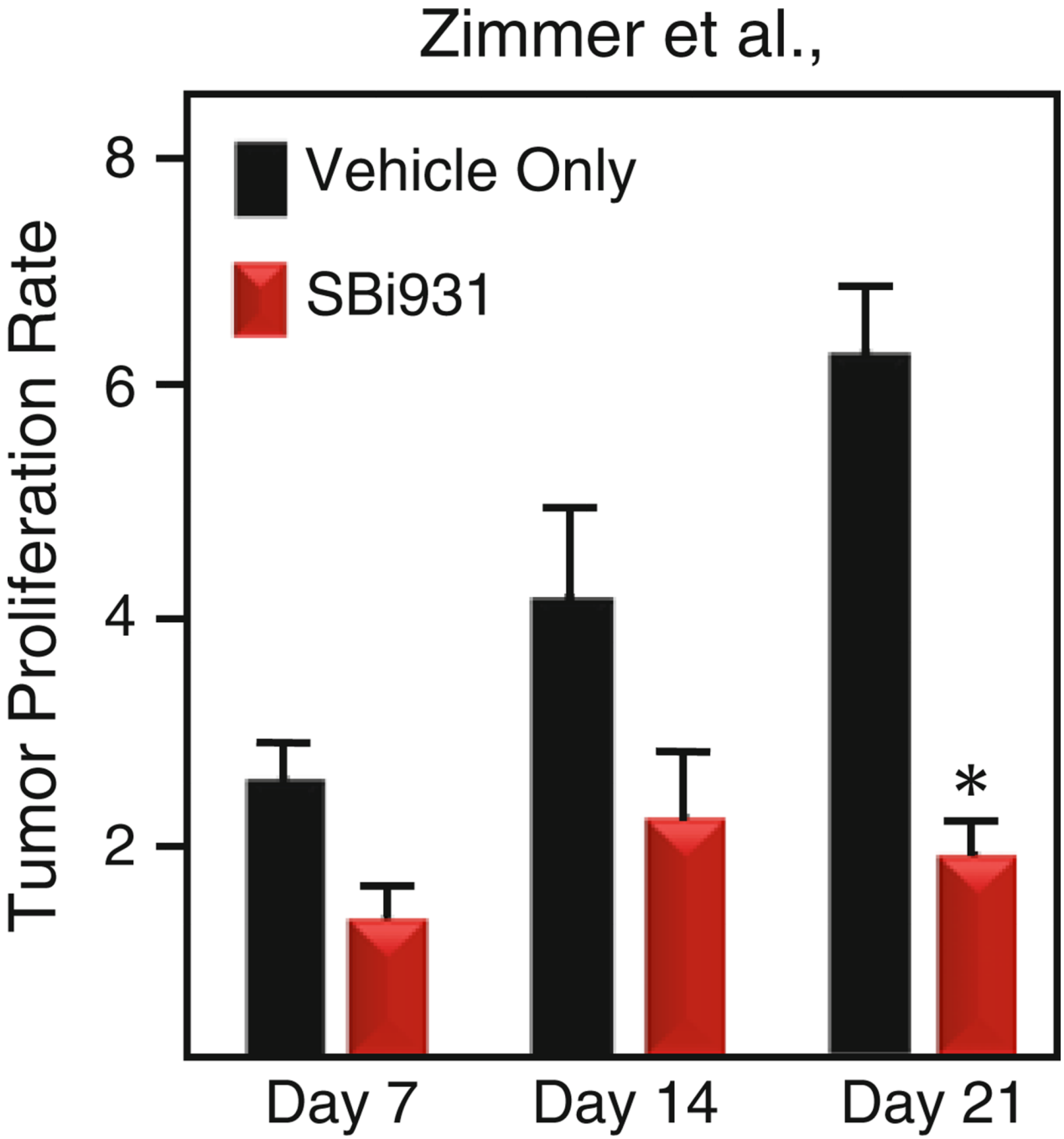

Figure 2.

Intratumoral in vivo screening trial. The histogram depicts the mean tumor responsiveness for intratumoral injection of SBi931 (*red bars*) or vehicle only (*black bars*) every other day for 21 days. Asterisk denotes $p < 0.05$ when compared to vehicle only.

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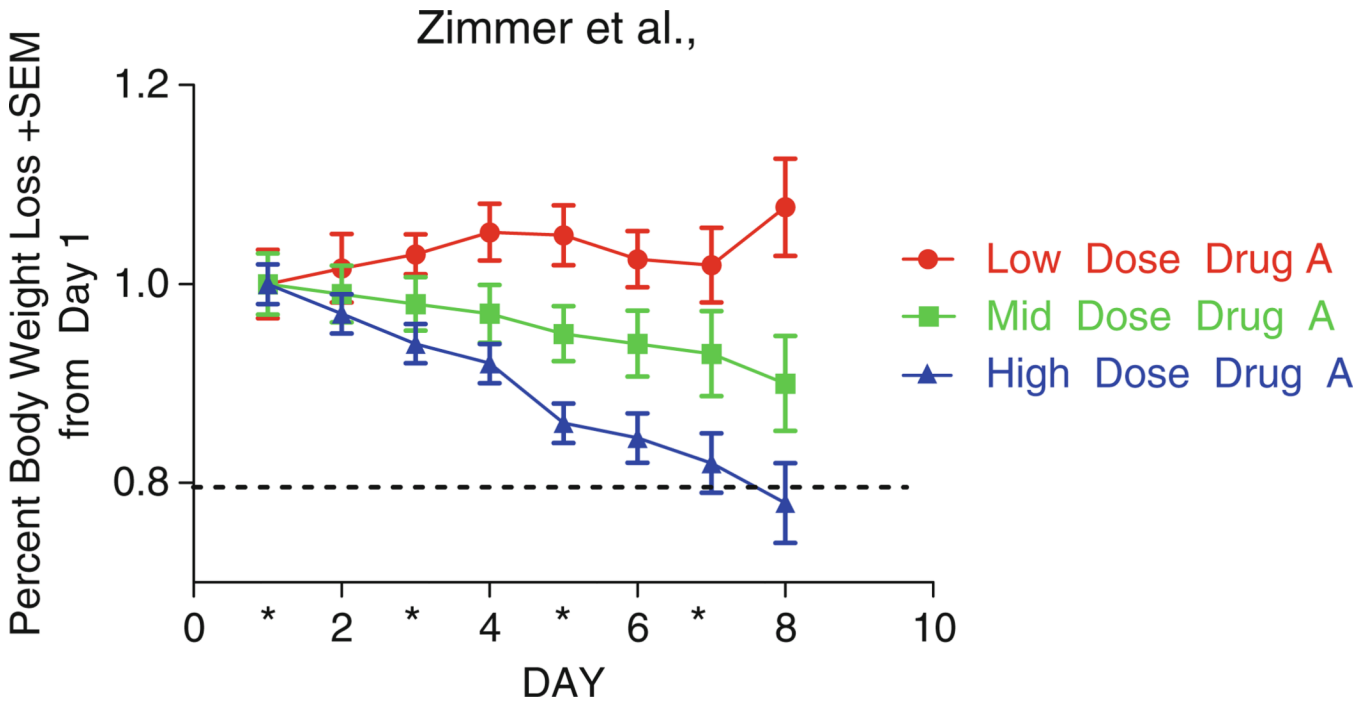

Figure 3.

Systemic tolerability testing/MTD. The graph depicts the percent body weight loss verses time for groups (n = 3 mice) receiving daily IP doses (mid, low, or high) of a drug for 7 consecutive days. The decrease in mean body weight in the high-dose group was greater than 20% indicating that this dose exceeds the MTD.